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Silver nanoparticles as a potential antimicrobial additive for weaned pigs

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ABSTRACT

Three experiments were carried out in order to determine the potential of silver nanoparticles as an additive in diets for weanling pigs. In Experiment 1, ileal contents of 4 pigs weaned 7 days before were incubated *in vitro* for 4 h at 37 °C with 0, 25, 50 and 100 μg Ag/g. Metallic silver (in colloidal form) linearly reduced coliforms (P=0.003) and lactobacilli (P=0.041) concentration, but did not affect the lactobacilli proportion compared with the control. In Experiment 2, three groups of 5 weaned pigs were given a diet with 0, 20 or 40 mg Ag/kg. The second week after weaning daily growth of pigs increased linearly (P=0.007) with the dose of silver nanoparticles. A trend (P=0.073) for a linear reduction in the ileal concentration of coliforms was observed by culture counts, but lactobacilli remained unaffected. There were no differences among treatments in the ileal concentration of coliforms or lactobacilli measured by FISH. However, the concentration of total bacteria (P=0.010) and *Atopobium* (P=0.001) decreased at a decreasing rate. No differences were detected in the other bacterial groups tested, except for a lowest concentration of the *Clostridium perfringens*/*Clostridium histolyticum* group in 20 mg Ag/kg (P=0.012). No treatment effect was detected in histological examination of ileal mucosa. In Experiment 3, productive performance and silver retention in tissues with 0, 20 or 40 mg Ag/kg diet were studied with 6 lots of 4 piglets per treatment in five weeks after weaning.

Abbreviations: aNDFom, neutral detergent fibre, exclusive of ashes and with amylase; FISH, fluorescence *in situ* hybridization; SEM, standard error of means.

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Feed intake was highest in treatment with 20 mg Ag/kg ($P<0.05$). No effect was observed on apparent digestibility coefficients. After 5 weeks, there was no silver retention in skeletal muscles or kidneys, but it was observed, although in minimal proportions, in liver (1.354 and 2.445 μg Ag/g dry liver for 20 and 40 mg Ag/kg). The potential effect of metallic silver as a dietary additive on intake and growth of weaned piglets could be mediated through its antimicrobial properties, either against certain bacterial groups or reducing the microbial load of the small intestine; however, other beneficial effects over the host metabolism cannot be discarded.

1. Introduction

In the last two decades, several types of additives have been proposed in pig production as alternatives to the use of antibiotics as growth promoters, such as organic acids, oligosaccharides, plant extracts or probiotics (Cowan, 1999; Naughton et al., 2001; Gardiner et al., 2004; Franco et al., 2005). However, their effect in preventing digestive disorders at weaning and promoting higher productive performances is variable and in most cases below the magnitude previously reached with the use of antibiotics.

Silver compounds are currently used as antimicrobial agents in a variety of applications, including coating of catheters, dental resin composites, burn wounds and homeopathic medicine, with a minimal risk of toxicity in humans (Lansdown, 2006). The small size of nanoparticles of metallic silver (below 200 nm) in solid or colloidal state allows for a higher microbiological effect than silver salts (Atiyeh et al., 2007). Besides, metallic silver is potentially less toxic (Wadhera and Fung, 2005) and it would be deactivated at a slower rate by gastric HCl (Atiyeh et al., 2007) than silver salts. In the 1950s, silver nanoparticles were used as additive in poultry nutrition, but the price could not compete with that of antibiotics. Nowadays, a more efficient industrial production process makes it potentially interesting as prebiotic.

Silver is toxic to microorganisms by poisoning respiratory enzymes and components of the microbial electron transport system, and it also binds to bacterial surface, altering the membrane function (Percival et al., 2005), and to DNA bases, thus inhibiting replication (Wright et al., 1994). Other metal compounds used as growth promoters in pig nutrition such as zinc oxide or copper sulphate also modulate digestive microbiota and reduce post-weaning diarrhoea (Jensen-Waern et al., 1998; Broom et al., 2006), but their role on the gut microbial ecosystem (Hojberg et al., 2005), in enhancing activity of pancreatic enzymes or in maintaining intestinal morphology (Hedemann et al., 2006) is not yet clear. There is no information available about negative effects of silver on pigs in productive conditions. The minimum silver level observed to have an adverse effect on chicken, rat and mice has been 300 μg/kg feed; on this basis, NRC (1980) set the maximum tolerable level of silver for poultry and swine at 100 μg/kg feed.

The current study studied the effect of different dietary doses of metallic silver nanoparticles in diets for weaned pigs on the digestive microbiota and gut morphology (Experiments 1 and 2), and in productive performances and silver retention in tissues (Experiment 3).

2. Materials and methods

2.1. Experiment 1: Effect on digestive microbiota in vitro

An initial experiment was carried out *in vitro* to estimate the dose effect of metallic silver on digestive microbiota of weaned piglets. Four 21-day-old (Pietrain x Large White) × (Large White × Landrace) piglets were used as donors of inoculum. Animals were weaned and received a mixed feed as meal, whose ingredient and chemical composition is shown in Table 1 as diet 0–2 weeks after weaning. After 7 days of *ad libitum* feeding, pigs were slaughtered after previous stunning with CO_2 and after three hours of feed and water deprivation (protocol approved by the Comisión Ética Asesora

Table 1
Ingredient and chemical composition of the piglet diets for the first two weeks after weaning (Experiments 1–3) and for 3–5 weeks (Experiment 3) after weaning.

	0–2 Weeks	3–5 Weeks
Ingredients (g/kg)		
Extruded cereal	580	–
Maize grain	–	400
Barley grain	–	250
Soybean meal	180	200
Whey powder	120	60
Fishmeal LT	70	40
Sunflower oil	5	15
L-Lysine 50	5	–
Dicalcium phosphate	20	18
Calcium carbonate	12	10
Sodium chloride	4	3
Mineral–vitamin mix[a]	4	4
Chemical composition (g/kg dry matter)		
Organic matter	908	923
Crude protein	197	189
Ether extract	23	43
Neutral detergent fibre	120	175

[a] To give per kg feed: 13,000 IU vit. A; 2500 IU vit. D3; 13 mg vit. E; 1.5 mg vit. K; 5 mg riboflavin; 1 mg tiamin; 2.2 mg vit. B6; 0.02 mg vit. B12; 25 mg niacin; 10 mg calcium pantothenate; 200 mg choline chloride; 110 mg Zn; 50 mg Mn; 100 mg Fe; 165 mg Cu; 0.5 mg Co; 0.22 mg Se; 0.5 mg I.

de Experimentación Animal of the University of Zaragoza). The ileal contents were sampled and immediately transferred to the laboratory where 1 ml aliquots were used to inoculate duplicate tubes with 3 ml of 0.02 M phosphate buffer at pH 6.0 and metallic silver (in colloidal form) to reach a final concentration of 0, 25, 50 and 100 μg Ag/g, resulting in two tubes of each silver dose per each pig. All tubes were added with 0.04 g of pig faeces to increase the microbial concentration. Tubes were incubated at 37 °C for 4 h and used for the culture of digestive coliform and lactobacilli bacteria.

2.2. Experiment 2: Digestive microbiota and gut morphology

Fifteen 28-day-old male piglets of the same crossbreed as in Experiment 1 and weighing on average 7.7 ± 0.16 kg were used. Animals were obtained from a commercial farm, where they had received creep feed from day 14. Pigs were weaned on day 28 and randomly distributed to three experimental treatments, resulting five piglets per group. Throughout the 14 days of experimental period, pigs were placed in 2 m × 2 m pens provided with plastic slatted floor and an automatic drinking device in a temperature-controlled barn (23–25 °C). Piglets were individually weighed on days 7 and 14 of the experimental period.

Animals were given the same basal diet as in Experiment 1 (Table 1), unsupplemented (control) or supplemented with Argenta (Laboratorios Argenol S.L., Zaragoza, Spain). This product consists of silver nanoparticles (minimum 0.80 particles between 60 and 100 nm) in sepiolite as carrier (10 g Ag/kg), that was added to get a final concentration of 20 and 40 mg Ag/kg feed in fresh matter basis. Daily feed for each pen was given *ad libitum*, and feed refusals were weekly weighed.

On day 14, animals were slaughtered after stunning with CO_2 as detailed in Experiment 1. The entire gastrointestinal tract was removed and the last 75 cm of the small intestine was separated, longitudinally excised and its content sampled. One sample (2 ml) was immediately frozen in liquid N and stored at −80 °C until its molecular microbiological enumeration, a second sample (2 ml) was refrigerated and processed in less than 1 h for microbiological cultures and a third sample (10 ml) was immediately processed for starch fermentation activity. Finally, one segment (approximately 10 cm) from this portion of the small intestine was cut, rinsed in ice-cold 10% phosphate buffered formalin (pH 7.4) and stored for histological examination.

2.3. Experiment 3: Productive parameters and silver retention in tissues

Seventy-two 21-day-old male piglets (6.3 ± 0.15 kg initial weight) of the same origin and management as in Experiment 2 were weaned and allocated by weight in 6 blocks of increasing weight and distributed within each block to three experimental treatments in a randomised block design, resulting in six pen replicates of four piglets per treatment. Each group of animals was placed in an individual pen in the same conditions as in Experiment 2. Animals received a basal diet formulated to meet the requirements of weaned piglets for the first two weeks (the same as in Experiment 2) and for weeks 3–5 after weaning (INRA, 1989). Ingredient and chemical composition is shown in Table 1. Feed was given *ad libitum*, according to the visual control of the previous day refusals. Four days before the change of diet (on days 10 and 31 of the experimental period) 1 g chromic oxide per kg feed was daily added as digestibility marker.

Experimental treatments consisted of the basal diet given unsupplemented (control) or with 20 or 40 mg Ag/kg as silver nanoparticles (Argenta). Pig weight and feed refusals were weekly controlled per replicate. Daily pig growth was estimated by linear regression analysis of weight on time. At the end of weeks 2 and 5 (days 14 and 35) fresh faeces samples were taken at 09:00 and 16:00 h from one pig from each pen (6 animals per treatment) for the determination of diet digestibility. The chosen piglets were isolated in individual pens for about 30 min and the faeces taken from the floor immediately after excretion. Faecal samples were dried (60 °C, 48 h), pooled and stored until chromium determination.

At the end of the experiment (day 35) three pigs from each pen of treatments including metallic silver (20 and 40 mg Ag/kg; $n = 36$) were slaughtered in the same conditions as in Experiment 2. Representative samples of liver, kidney and skeletal muscle tissue (*biceps femoralis*) were collected and dried at 60 °C for 72 h before being analysed for silver concentration.

2.4. Chemical, microbiological and histological analyses

The feed was sampled daily and pooled weekly for the analysis of dry matter, organic matter, crude protein and ether extract according to the Association of Official Analytical Chemists (1995) methods, and neutral detergent fibre (aNDFom) following the Van Soest et al. (1991) procedure.

Chromium concentration from feeds and daily pooled faecal samples were analysed by AES-ICP after hydrolysis with nitric:perchloric acids (5:1), as described by Vega and Poppi (1997). For silver determination, samples (5 g) of dried organs were defatted with 35 ml petroleum ether at reflux in a Soxtec 1043 apparatus (Foss Tecator, Sweden), ashed at 650 °C for 8 h and hydrolysed with 10 ml of 50% nitric acid for 1 h at 100 °C. After complete digestion, the residue was filtrated, made up to 25 ml with distilled water and analysed in the same equipment as chromium.

For the bacterial cultures, samples were serially diluted in sterile saline and spread on MacConkey and Rogosa agar media plates (PANREAC, Barcelona, Spain), that were incubated at 37 °C for 24 and 48 h, respectively, for the culture of coliforms (*Escherichia coli, Enterococcus faecalis* and *Salmonella enteritidis*) and lactobacilli (*Lactobacillus* spp.), respectively. During lactobacilli culture, atmosphere was enriched with 10% CO_2. Results were expressed on a logarithmic basis.

For microbial enumeration using FISH, ileal contents were diluted 1/10 (w/v) in sterile ice-cold PBS (0.1 M, pH 7.0), homogenised and centrifuged (1500 × *g* for 3 min) to remove particulate matter and fixed for 4 h with 4% (w/v) paraformaldehyde (pH 7.2) at 4 °C. Samples were washed twice in sterile PBS and resuspended in PBS/ethanol at −20 °C and stored. The hybridisation was carried out as described by Ames et al. (1999) with genus- and group-specific 16S rRNA-targeted oligonucleotide probes: Bif164 (Manz et al., 1996), Bac303 (Langendijk et al., 1995), Chis150 (Franks et al., 1998), Erec482 (Franks et al., 1998), Lab158 (Harmsen et al., 1999), Sal3 (Nordentoft et al., 1997), Prop853 (Walker et al., 2005), Ato291 (Harmsen et al., 2000) and Eco1531 (Poulsen et al., 1994), that were specific for *Bifidobacterium*, *Bacteroides*/*Prevotella* subgroup, *C.perfringens*/C. *histolyticum* subgroup, *C. coccoides*/*Eubacterium rectale* group, *Lactobacillus*/*Enterococcus* spp., *Salmonella*, *Clostridium* cluster IX (including *Selenomonas*, *Veillonella*, *Megasphaera* and Mitsuokella), *Atopobium* cluster (including *Coriobacteriaceae* spp.) and *E. coli*, respectively. The nucleic acid stain 4,6-diamidino-2-phenylindole (DAPI) was used for total cell counts. DNA probes were tagged with a Cy3 fluorescence dye such that the hybridised samples could be examined using fluorescence microscopy. The stored cell suspensions were added to a six-well Teflon

slide used as hybridisation supports, dried at 46 °C (15 min) and dehydrated with ethanol (50, 80 and 96%, 3 min). After drying, 50 μl of hybridisation buffer containing 5 ng/μl of probes was added. The slides were incubated in a humid chamber at appropriate temperatures for each probe during 4 h and washed for 15 min. After air drying, a stop solution was added and a cover slide applied, leaving the samples overnight at room temperature in dark conditions. Counts were expressed as 10 Log (no./g dry digesta). When a bacterial type in a sample was not detected, the detection limit (17,9531.2 bacteria/g wet sample) was assumed.

For histological examination, samples of intestinal mucosa were stained with haematoxylin and eosin, and seven well-orientated villi and their adjacent crypts were measured under the microscope (50× magnification) using an eyepiece micrometer, in order to calculate mean villous height and crypt depth.

2.5. Statistical analysis

Results were analysed using the Statistix 8 software package (Analytical Software, 2003). Data from Experiment 1 were analysed as a randomised block design considering the average of the two samples for each silver concentration as the experimental unit, and each animal as a block. In Experiment 2, the effect of silver dose was studied as a one-way ANOVA. In Experiment 3, productive parameters and digestibility data in weeks 0–2 and 3–5 after weaning were analysed separately as randomised block designs, considering the replicate (initial weight) as a block. Orthogonal polynomials were used to split the treatments effect into linear, quadratic and cubic responses. Differences among means with $P<0.05$ and $0.05<P<0.10$ were accepted as representing statistically significant differences and tendencies for differences, respectively.

3. Results

3.1. Experiment 1

Average concentration of coliforms and lactobacilli for the different concentrations of metallic silver is shown in Table 2. Total concentration of coliforms was linearly reduced with the concentration of metallic silver in the medium ($P=0.003$). Similarly, coliform proportions regarding the control (without silver) were linearly reduced by silver concentration ($P=0.046$). A linear reduction in the concentration of lactobacilli was also observed ($P=0.041$), but no effect was observed in the proportion of lactobacilli with respect to the treatment without silver ($P>0.10$).

3.2. Experiment 2

Daily individual intake estimated from total group intake was, on average, 161, 143 and 177 g DM/d and 257, 313 and 365 g DM/d for the control, 20 and 40 mg Ag/kg from 0 to 7 and 8 to 14 days

Table 2
Concentration (10 Log(no./ml)) and relative proportion of cultured bacteria in MacConkey (coliforms) and Rogosa (lactobacilli) agar from the ileal content of pigs cultured *in vitro* with 0, 25, 50 or 100 mg of metallic silver per kg (Experiment 1).

	0	25	50	100	SEM	Linear	Quadratic
Coliforms							
Concentration	5.8	5.5	5.3	5.1	0.12	**	NS
Proportion[a]	–	0.96	0.92	0.88	0.021	*	NS
Lactobacilli							
Concentration	6.0	5.7	5.5	5.5	0.14	*	NS
Proportion	–	0.95	0.92	0.91	0.024	NS	NS

NS: P>0.10.
[a] Proportion is estimated considering bacterial concentration with 0 mg Ag/kg as 1.
* P<0.05.
** P<0.01.

Table 3
Average individual daily weight gain (g/d) and concentration (10 Log(no./ml)) of cultured bacteria in MacConkey (coliforms) and Rogosa (lactobacilli) agar and fermentative activity (ml gas/g) after 24 h incubation of starch or xylan from the ileal content of pigs given the diet unsupplemented (control) or with 20 or 40 mg/kg of silver nanoparticles (Experiment 2).

	Control	20	40	SEM	Linear	Quadratic
Daily weight gain						
0–7 days	107	122	157	41.3	NS	NS
7–14 days	314	393	461	36.4	**	NS
Coliforms	5.2	5.0	4.7	0.18	T	NS
Lactobacilli	6.1	6.5	6.1	0.32	NS	NS
Lactobacilli/coliform ratio	1.18	1.26	1.31	0.094	NS	NS
Starch fermentation	181	176	170	7.3	NS	NS
Xylan fermentation	58	78	57	13.4	NS	NS

T: P<0.10; NS: P>0.10.
** P<0.01.

after weaning, respectively. Individual daily growth of piglets did not differ among treatments in the first week, but increased linearly (P=0.007) with the addition of silver nanoparticles in the second week.

Average concentration of cultured coliforms and lactobacilli and bacterial activity of ileal contents against starch and xylan are shown in Table 3. A trend (P=0.073) for a linear reduction of coliform concentration was observed as supplementation of silver nanoparticles increased. No significant effect of silver supplementation was observed on the other studied parameters.

Bacterial composition in the ileal contents of pigs analyzed by FISH is shown in Table 4. Total bacterial concentration estimated by DAPI was lowest when given 20 mg Ag/kg (P=0.010). Coverage (considered as the proportion of total bacteria accounted for the different types studied) was very variable between animals, but it reached more than 0.75 in treatments with 40 and 20 mg Ag/kg. Bacteria from the *Bifidobacterium* group were detected in three out of five pigs from the control treatment, and in only one pig from the treatments including silver nanoparticles. Therefore, this bacterial group was not considered for statistical studies. Concerning the other bacterial groups, a quadratic effect was manifested in the *C. perfringens*/*C. histolyticum* group (P=0.012), with the lowest numbers recorded for 20 mg Ag/kg. Bacterial concentration in the *Atopobium* cluster decreased at a decreasing rate (P<0.001). It is worth noting that bacteria from the former group were not detected in one animal from the control treatment and in four out of five piglets from the treatment with 20 mg Ag/kg.

The results from the histological study of the ileal mucosa of piglets two weeks after weaning are shown in Table 5. No effect of silver addition was detected for villous height, crypt depth or the height to depth ratio.

Table 4
Average concentration (10 Log(no./g dry digesta) of total bacteria and bacterial types together with coverage proportion, from the ileal contents of pigs given the diet unsupplemented (control) or with 20 or 40 mg/kg of silver nanoparticles (Experiment 2).

	Control	20	40	SEM	Linear	Quadratic
Total bacteria	9.9	9.2	9.5	0.12	T	**
Bacteroides-Prevotella	8.6	8.7	8.0	0.45	NS	NS
C. perfringens/C. *histolyticum*	7.2	6.5	7.9	0.27	NS	**
C. coccoides/*Eubacterium rectale*	7.4	6.9	6.8	0.23	NS	NS
Lactobacillus/*Enterococcus*	9.0	8.6	8.6	0.33	NS	NS
Salmonella	7.6	7.2	7.2	0.31	NS	NS
Atopobium	8.3	7.0	7.2	0.10	***	***
E. coli	7.9	8.5	8.5	0.25	NS	NS
Coverage	0.37	0.92	0.77	0.168	NS	NS

T: P<0.10; NS: P>0.10.
** P<0.01.
*** P<0.001.

Table 5
Average villous height (μm), crypt depth (μm) and height to depth ratio in the ileal mucosa of pigs given the diet unsupplemented (control) or with 20 or 40 mg/kg of silver nanoparticles (Experiment 2).

	Control	20	40	SEM	Linear	Quadratic
Villous height	356	397	341	24.4	NS	NS
Crypt depth	190	191	159	12.3	T	NS
Height/depth ratio	1.90	2.09	2.21	0.208	NS	NS

T: P<0.10; NS: P>0.10.

Table 6
Productive performance (dry matter intake, daily weight gain and feed to gain ratio) and dry matter and organic matter apparent digestibility coefficients (DMD and OMD) of piglets receiving the basal diet unsupplemented (control) or with 20 or 40 mg/kg of silver nanoparticles, for 0–2 weeks and 3–5 weeks after weaning (Experiment 3).

	Control	20	40	SEM	Linear	Quadratic
Intake (g DM/d)						
0–2 weeks	154	189	148	8.5	NS	**
3–5 weeks	527	670	630	32.3	*	*
Daily gain (g/d)						
0–2 weeks	66	102	93	11.0	NS	NS
3–5 weeks	337	375	347	21.2	NS	NS
Feed to gain (kg/kg)						
0–2 weeks	2.1	1.9	1.7	0.20	NS	NS
3–5 weeks	1.6	1.8	1.8	0.05	***	*
DMD						
0–2 weeks	0.81	0.85	0.83	0.013	NS	NS
3–5 weeks	0.84	0.83	0.83	0.014	NS	NS
OMD						
0–2 weeks	0.84	0.87	0.85	0.011	NS	NS
3–5 weeks	0.85	0.84	0.85	0.012	NS	NS

NS: P>0.10.
* P<0.05.
** P<0.01.
*** P<0.0015.

3.3. Experiment 3

No severe cases of diarrhoea (apparent liquid faeces) were observed during the experiment, and when milder cases appeared it was irrespective of the treatment and the pigs recovered in one or two days. Productive performances (average daily gain, daily feed intake and feed to gain ratio) for weeks 0–2 and 3–5 after weaning are shown in Table 6. Feed intake was highest for the treatment with 20 mg Ag/kg for 0–2 weeks (P=0.006) and increased at a decreasing rate for 3–5 weeks (P=0.048). The feed to gain ratio increased at a decreasing rate in weeks 3–5 (P=0.026). The experimental treatment did not affect apparent digestibility coefficients in any period.

Analysis of silver concentration in tissues of pigs given feed supplemented with 20 or 40 mg/kg of metallic silver for 5 weeks showed a lack of silver deposition in skeletal muscle or kidney in any of the studied cases ($n = 18$ per treatment). In contrast, this metal was detected in the livers of all silver-supplemented animals, in average concentrations of 1.35 and 2.45 μg Ag/g dry liver for 20 and 40 mg Ag/kg treatments (maximum levels of 1.01 and 2.30 μg/g, respectively), equivalent to 0.435 and 0.837 μg/g wet tissue.

4. Discussion

To the author's knowledge, there are no data available on the use of silver nanoparticles as feed additive in pig diets. The antimicrobial activity of silver is manifested by blocking the electron transport

system, altering the function of the bacterial membrane and inhibiting the DNA replication (Wright et al., 1994; Percival et al., 2005). Although these effects have been demonstrated experimentally against *E. coli* (Zhao and Stevens, 1998; Sondi and Salopek-Sondi, 2004), our first concern was to check if silver could also affect other potentially beneficial gut bacteria, such as lactobacilli. Other metals such as zinc and copper have been widely used (Hahn and Baker, 1993; Smith et al., 1997), and it can be expected that their action on pig growth and digestive bacterial community could be similar to some extent; therefore, considering the lack of data on silver addition, their effects are used as discussion arguments here. Culture counts from Experiment 1 confirmed a linear decrease of coliforms with silver concentration, while the effect on lactobacilli was not so clearly manifested. However, it has to be considered that diarrhoea is a multifactorial process, and not all species/strains of the coliform group are potentially harmful for the host; indeed, a competitive inhibition for receptor sites by non-pathogenic strains of *E. coli* could decrease adhesion of pathogens, and thus a high diversity within the coliform group, rather than a low coliform concentration, would better prevent pathological digestive processes (Katouli et al., 1999). Moreover, the wide enteric bacterial diversity ensures that not only lactobacilli and coliforms are potentially beneficial or harmful, respectively, for pig health status. Once the selectiveness of the response of digestive bacteria was confirmed, silver dietary concentrations in Experiments 2 and 3 were established fixing the maximum level of inclusion based on economical viability.

Observed coverage by FISH analyses was within (control treatment) or higher (20 and 40 mg Ag/kg treatments) than that obtained in the jejunum by Castillo et al. (2007). The concentration of other beneficial bacterial groups analysed was not affected by silver supplementation, since the presence of minimal proportions of *Bifidobacterium* organisms was detected only in four animals (three from the control group and one receiving 20 mg Ag/kg), and concentration of *C. coccoides*/*Eubacterium* group (detected by Erec482), that could be beneficial for the piglet because of their production of short chain fatty acids, was unaffected. In general, there were minor changes in the concentration of the different bacterial groups tested by FISH, showing a reduction of organisms from the *Atopobium* group with silver supplementation, and a lower proportion of the potentially harmful *Clostridium hystoliticum* group (detected by Chis150) with 20 compared to 40 mg/kg of silver nanoparticles. Bacteria from the *Atopobium* cluster have been observed in relatively high concentations in human babies, reducing their numbers with age (Harmsen et al., 2000), although their metabolic role is still unclear.

In contrast to *in vitro* results from Experiment 1 and those from culture analysis in Experiment 2, no significant effects of silver nanoparticles on coliforms were observed in FISH analyses from Experiment 2. Despite an antibacterial effect of zinc in weaned pig diets is generally assumed, thus reducing the incidence of diarrhoea, a lack of response in intestinal or faecal concentration of coliforms or other bacterial groups to 2500–3100 mg/kg of zinc supplementation has also been reported (Jensen-Waern et al., 1998; Li et al., 2001; Broom et al., 2006), although the inclusion of zinc in diets is restricted to below 150 ppm in Europe (EC 1831/2003). Hojberg et al. (2005) even reported an increase in coliforms and a reduction of lactobacilli when adding 2500 mg Zn/kg. These authors suggested that the reduction of gut commensal bacteria would reduce nutrient fermentation, thus rendering more available energy for the host. In this sense, our results also show a reduction in the concentration of total ileal bacteria (DAPI) with 20 mg Ag/kg (Table 4), although the extent of *in vitro* microbial fermentation of starch and xylan was not affected (Table 3). In any case, despite their potential negative impact on intestinal ecosystem, all bacteria compete with the host for nutrients; therefore, a high total bacterial concentration, even high numbers of *Atopobium, C. coccoides/E. rectale* or *Clostridium* cluster IX groups, would indicate a lower availability of nutrients to be absorbed at the distal half of the small intestine. These results suggest that either there was no clear effect of silver nanoparticles on gut microbiota and therefore the beneficial effects would be related with other metabolic aspects, or that changes in bacterial diversity within the microbial groups would justify different productive performances.

It is difficult to explain the different microbial responses to silver addition *in vitro* (Experiment 1) and *in vivo* (Experiment 2). One possibility would be that metallic silver particles can be partly converted into AgCl while passing through the stomach, thus reducing their antimicrobial properties (Atiyeh et al., 2007). However, this hardly occurs with metallic silver, and in fact major effect of HCl against silver nanoparticles when attacking silver sepiolite has been detected at pH 3 for 2 h *in vitro* (data not shown). It might also be that a certain proportion of silver would be absorbed through the

small intestine before reaching the ileum, but in such case it would be a minor proportion, considering the silver retention in tissues. Anyway, the lack of a marked response at a microbial level would indicate that other systemic effects could also be implicated in the response, as it has also been suggested for zinc (Zhou et al., 1994; Carlson et al., 1999).

No major effect of silver nanoparticles was observed on intestinal histology. Li et al. (2001) reported an increase in villous height by addition of zinc oxide to weanling pig diet, but this effect has not been recorded by others (Hedemann et al., 2006). Morphological changes in intestinal mucosa after weaning, and consequently the extent of beneficial effect of the additive in preventing/minimising these changes, are more prominent when weaning takes place earlier and in the first week after weaning (Hampson, 1986; Pluske et al., 1997), and this would explain the lack of response in this work.

The recorded growth rate in Experiment 3 was lower than expected for this type of diet and production conditions. Results were in the range of those observed by Zhang and Guo (2007) but were lower than those previously recorded in our facilities with similar animals and diets (Fondevila et al., 2001; Blasco et al., 2005). This is a consequence of the low daily intake, as is shown by the feed to gain ratios that fit with those observed in intensive production of weanling pigs. This experiment was carried out during the summer, and a technical problem in the temperature control system of the barn made the indoor temperature often over 30 °C, thus reducing daily intake of all pigs. In any case, feed intake increased when 20 mg Ag/kg was added to the basal feed, and despite the 1.6 times higher growth observed with 20 mg Ag/kg than with the control was not significant, the linear increase in growth rate of pigs when given 20 and 40 mg Ag/kg in the second week of Experiment 2 (Table 3) supports this hypothesis.

There was no effect of silver on apparent digestibility. As with silver, there are no references of a clear effect of zinc oxide when given as antimicrobial on apparent digestibility of diets for weaned pigs.

It has been reported that a high (from 95 to 300 mg Ag/kg, corresponding to 2.4–7.5-fold the maximum dose studied in this work) and chronic (applied for up to 18 weeks) dose of silver (in salts) may lead to a decreased weight gain in mice (Rungby and Danscher, 1984) and turkeys (Jensen et al., 1974). In some human individuals, these conditions may lead to its deposition in the skin, eye and occasionally in organs such as kidney or liver, although pathological symptoms are mostly cutaneous, and very rarely threaten organ function (Lansdown, 2006). These effects are mostly associated to the use of ionic rather than metal silver (Wadhera and Fung, 2005). The possible absorption and retention of metal silver in pig organs was also evaluated in this paper, despite the low dose of silver used and the reduced time of dietary inclusion (5 weeks). In this sense, it is worth noting that there were no traces of silver retained in skeletal muscle or in kidneys, and the retention in liver was only 0.84 mg/kg liver for the 40 mg Ag/kg treatment. This would imply an absolute retention of 0.335 mg Ag (assuming a liver weight of 400 g) for a total silver intake of 612 mg in 5 weeks. These figures are more valuably considering that liver retention of Zn when used as growth promoter ranged from 220–230 (Jensen-Waern et al., 1998; Carlson et al., 1999) to 445 mg/kg (Zhang and Guo, 2007) when 2250–3000 mg Zn/kg (as ZnO), respectively, were given.

5. Conclusions

Results from the present work indicate that low doses of metallic silver nanoparticles given as dietary additive could improve intake and growth of weaned piglets, although results from Experiment 3 do not allow to clearly state this. The effect of silver could be mediated through its antimicrobial properties, either by acting against certain bacterial groups or just reducing the microbial load of the small intestine; however, other beneficial effects over the host metabolism cannot be discarded. Silver retention in tissues is only appreciated at low levels in the livers of piglets, whereas it was not detected in kidneys or in skeletal muscles. Further studies would indicate whether the retained silver is eliminated during the fattening period, before reaching the market weight. The low concentration of silver included in weaned pigs' diets (20–40 mg Ag/kg), especially when compared with the currently used doses for other metals (250–350 mg Cu/kg or 2500–3000 mg Zn/kg) suggests a minimal environmental challenge of this metal.

In any case, this is a preliminary work on the use of metallic silver nanoparticles in diets for weaning piglets, and further research is needed to verify the obtained results, to clarify the microbiological and physiological mechanisms implicated and to contrast it with other additives.

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